Supporting Information

An ALP-activatable and mitochondria-targeted probe for prostate cancer-specific bimodal imaging and aggregation-enhanced photothermal therapy

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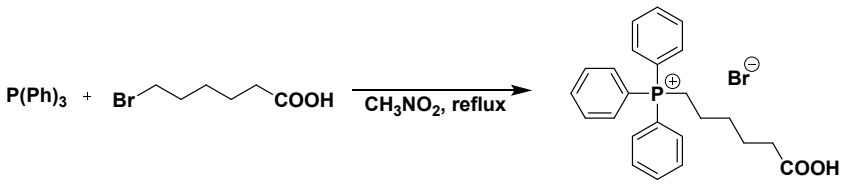
1. General information:

All reactions were carried out under an argon atmosphere using freshly distilled anhydrous solvents unless otherwise stated. All solvents were dried and distilled before use. Anhydrous dichloromethane (DCM) and dimethyl formamide (DMF) were distilled over $CaCl_2$ and CaH_2 respectively, and kept anhydrous with 4Å molecular sieves. The ultra-pure water was obtained by Millipore filtration system. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide Hydrochloride (EDCI) and N-Hydroxybenzotriazole (HOBt) were purchased from GL Biochem (Shanghai, China) Ltd. Alkaline phosphatase (ALP) was obtained from BaoMan Inc. (Shanghai, China) (one unit (U) is the enzyme activity that cleaves 1 μM of the standard substrate per minute at 37 °C). Other reagents and chemicals in AR grade were purchased from Adamas-beta® (Shanghai, China) Ltd and used without further purification.

The ^1H and ^{13}C NMR spectra were recorded at room temperature with Bruker AV 400 spectrometer. High-resolution mass spectra were obtained on an LCT Premier XE (electronic spray ionization, ESI) and a Waters Micromass® Q-TOF (ESI) spectrometer. The mass spectra of peptide were tested on an AB Sciex (MALDI-TOF) mass spectrometer. The analytical "High Performance Liquid Chromatography" (HPLC) was performed with the following parameters: reversed phase, RP-C_{18} HPLC column (10 μm particle size) and UV detector. The mobile phase was a gradient of 10-90% of methanol aqueous solution at a total flow rate of 0.8 mL/min. The UV absorption peaked at 400 nm of the elution was recorded for analysis. FT-IR spectra were taken on NICOLET 380 FT-IR, Thermo Electron Corp. The UV/Vis and fluorescence spectra were recorded with a Varian Cary 100 Conc UV-Visible Spectrometer and a Fluoromax-4 Spectrofluorometer (HORIBA Scientific), respectively. pH was measured by a Mettler Toledo FE 20K pH meter.

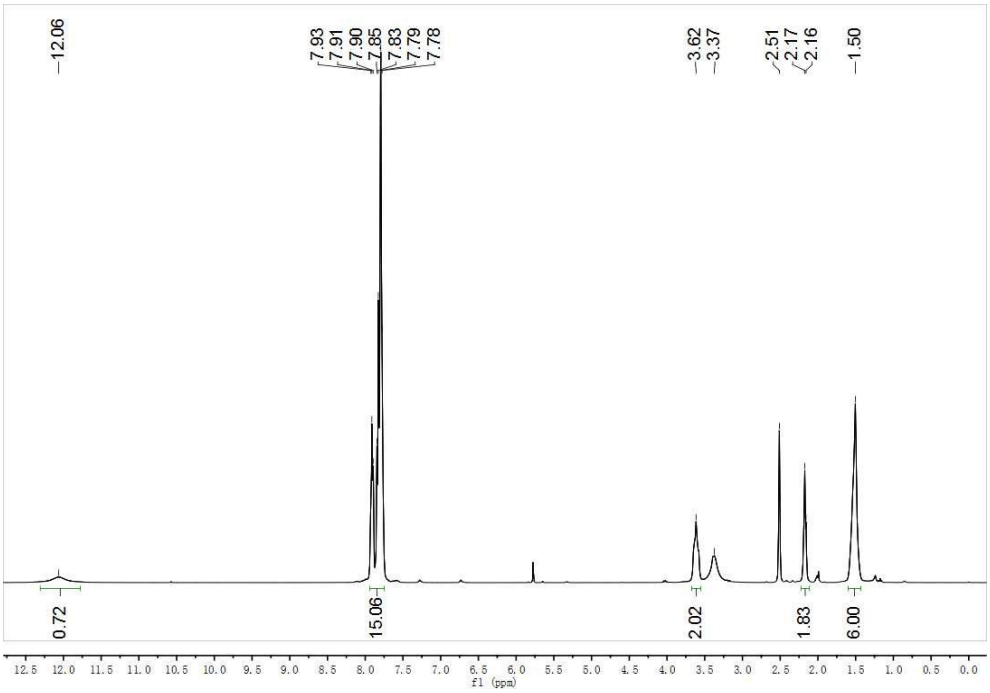
2. Synthesis of Enzyme-activatable Theranostic Probe (ETP):



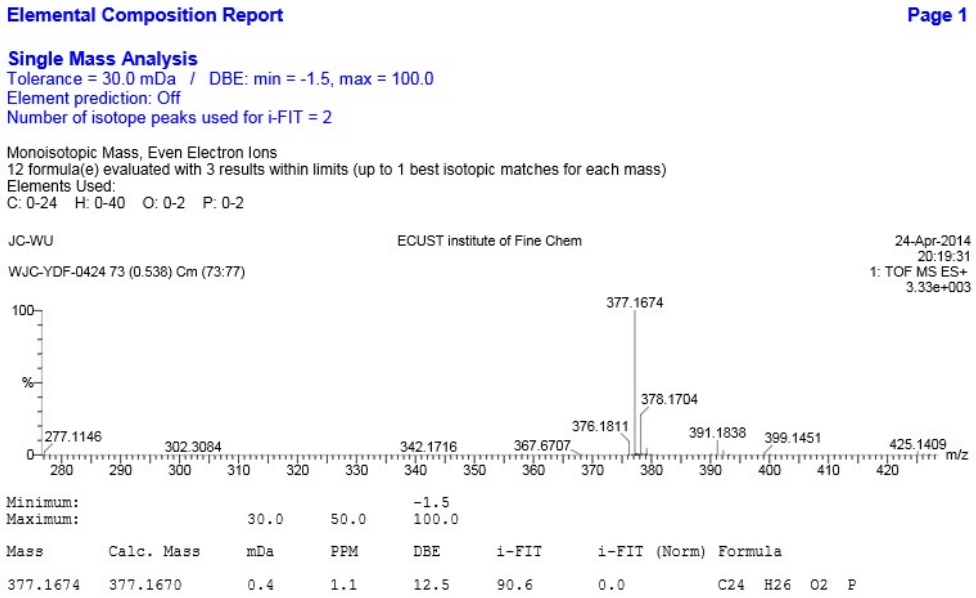
1

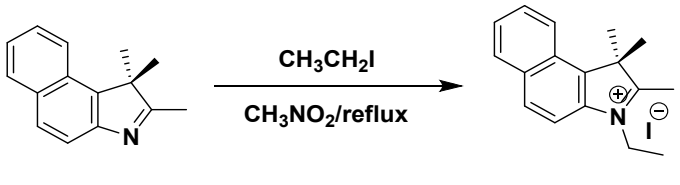
A mixture of 6-bromohexanoic acid (5.0 g, 25.8 mmol) and triphenylphosphine (7.4 g, 28.4 mmol) in 100 mL of nitromethane was refluxed for 12 h under argon. The mixture was allowed to cool at room temperature and concentrated in vacuum. The residue was crystallized from diethyl ether to give **1** as a white solid (11.7 g, 99.2 %). ^1H NMR (400 MHz, DMSO-d_6): δ = 1.50 (s, 8H), 2.17 (d, J = 6.5 Hz, 3H), 3.62 (s, 3H), 7.81 (m, 17H), 7.91 (m, 4H), 12.06 (s, 1H). HRMS-ESI (m/z): calcd for $C_{24}H_{28}BrO_2P$, 456.0854, found $[M\text{-}Br]^+$, 377.1674.

^{1}H NMR spectrum of compound **1** in DMSO-d_6:



ESI-MS spectrum of compound **1**:



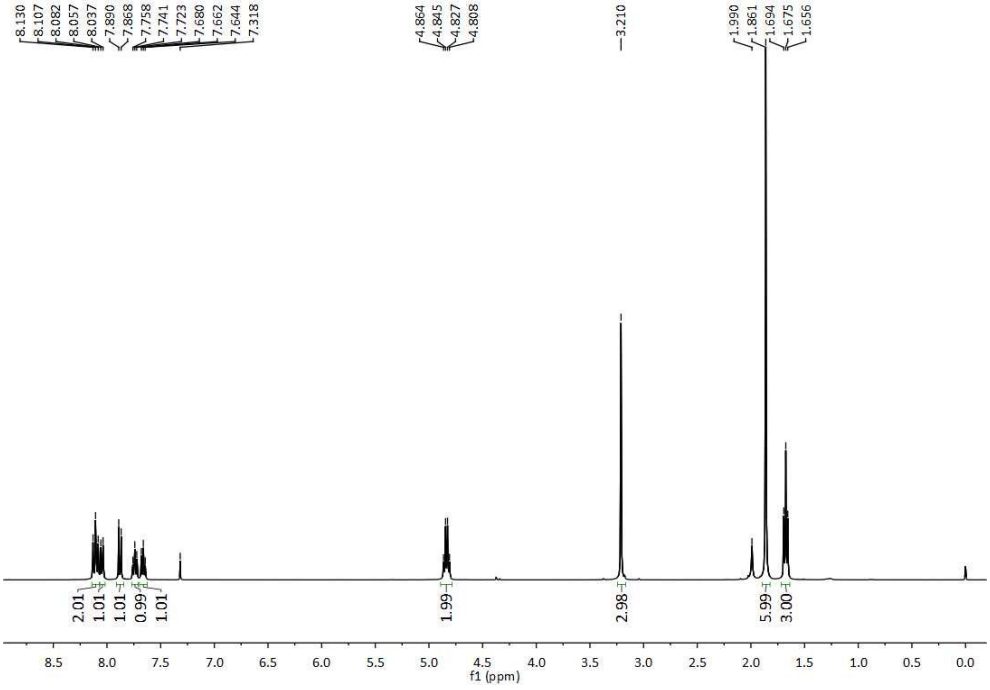


2

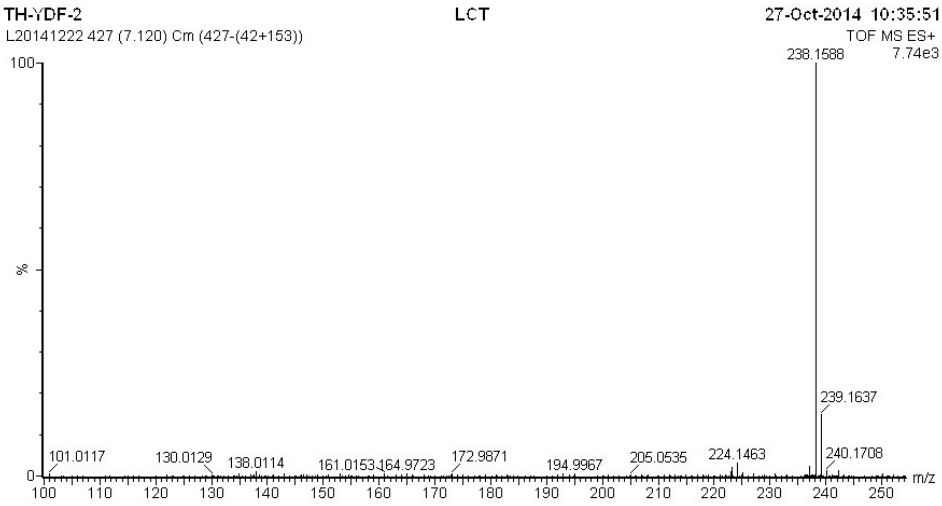
A suspension of 1, 1, 2-trimethyl-1h-benz[e]indol (10.0 g, 47.8 mmol) and iodoethane (13.3 mL, 167.2 mmol) in nitromethane (100 mL) was heated to reflux overnight. The heating was stopped and the solvent was cooled to room temperature. The precipitate was collected by filtration and

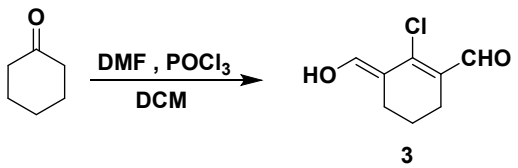
rinsed with ethanol (200 mL) to give **2** as a yellowish-green solid (12.3 g, 70.5 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.68 (t, *J* = 7.4 Hz, 3H), 1.86 (s, 6H), 3.21 (s, 3H), 4.84 (q, *J* = 7.4 Hz, 2H), 7.66 (t, *J* = 7.3 Hz, 1H), 7.74 (t, *J* = 7.1 Hz, 1H), 7.88 (d, *J* = 8.9 Hz, 1H), 8.06 (d, J = 8.2 Hz, 1H), 8.11 (t, *J* = 9.6 Hz, 2H). HRMS-ESI (m/z): calcd for $C_{17}H_{20}NI$, 365.0640, found $[M\text{-}I]^+$, 238.1588.

^{1}H NMR spectrum of compound **2** in DMSO-d_6:



ESI-MS spectrum of compound **2**:

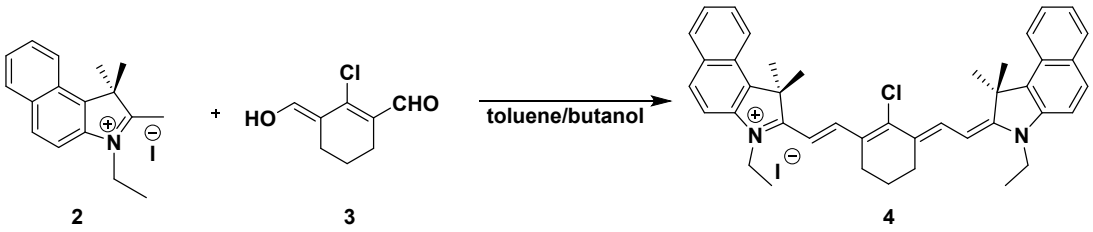




3

To a 250 mL three-necked flask containing dimethylformamide (40 mL) and methylene chloride (40 mL), phosphorus oxychloride (37 mL) in methylene chloride (35 mL) was added dropwise for

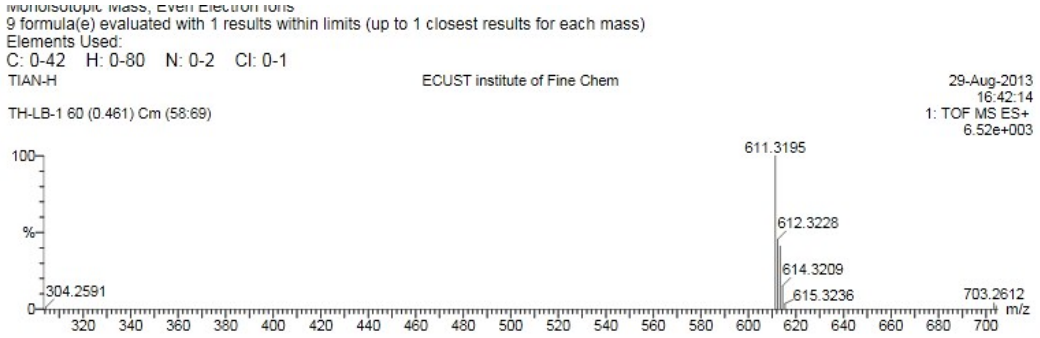
about 10 min. Then cyclohexanone (10.6 g) was added. The reaction mixture was refluxed for 3 h under an Ar atmosphere. The solvent was poured into ice, and the crude products were filtered and dried under vacuum to give crude intermediate product **3** (7.4 g, 40.5 %). The crude product was used for the next reaction without further purification.



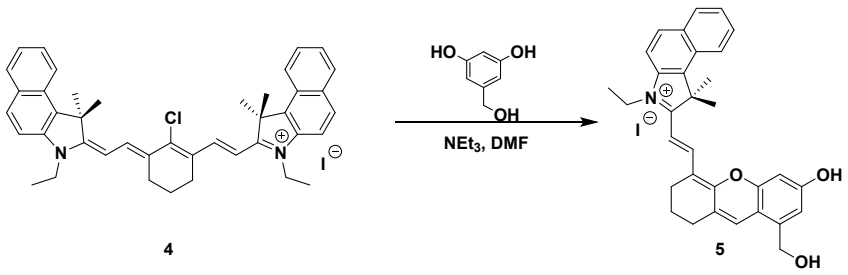
2 **3** **4**

A solution of **2** (1.5 g, 17.2 mmol), **3** (6.2 g, 8.5 mmol) in toluene (100 mL) and butanol (233 mL) was refluxed for 8 h, then treated with ether (60 mL). The solvent was evaporated, and the residue was purified by column chromatography (CH_2Cl_2:CH_3OH = 700:1 to 100:1) to give **4** as a solid (2.6 g, 33.8%). HRMS-ESI (m/z): calcd for $C_{42}H_{44}ClIN_2$, 739.1825, found $[M-I]^+$, 611.3195.

ESI-MS spectrum of compound **4**:



Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
611.3195	611.3193	0.2	0.3	21.5	9.6	0.0	C42 H44 N2 Cl

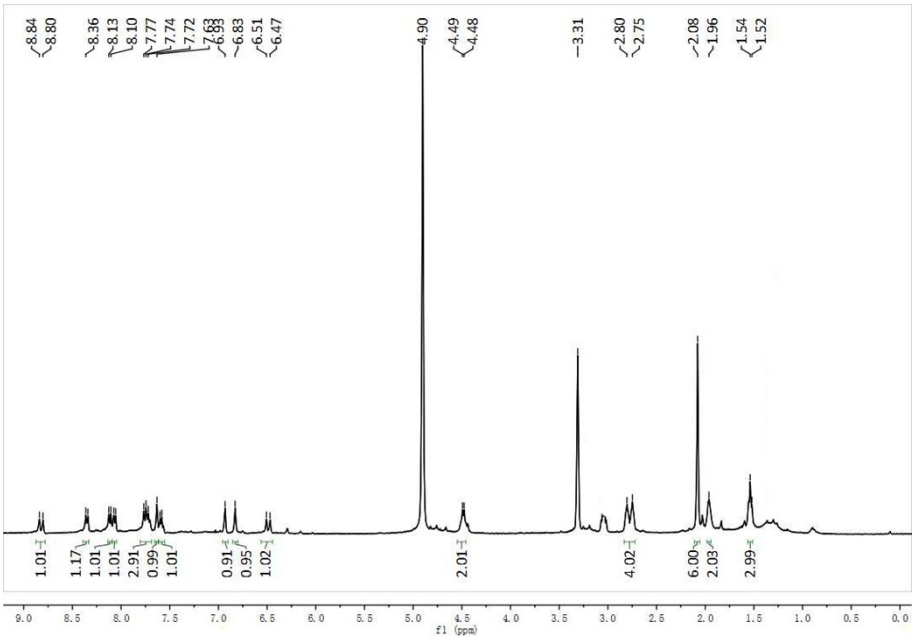


4 **5**

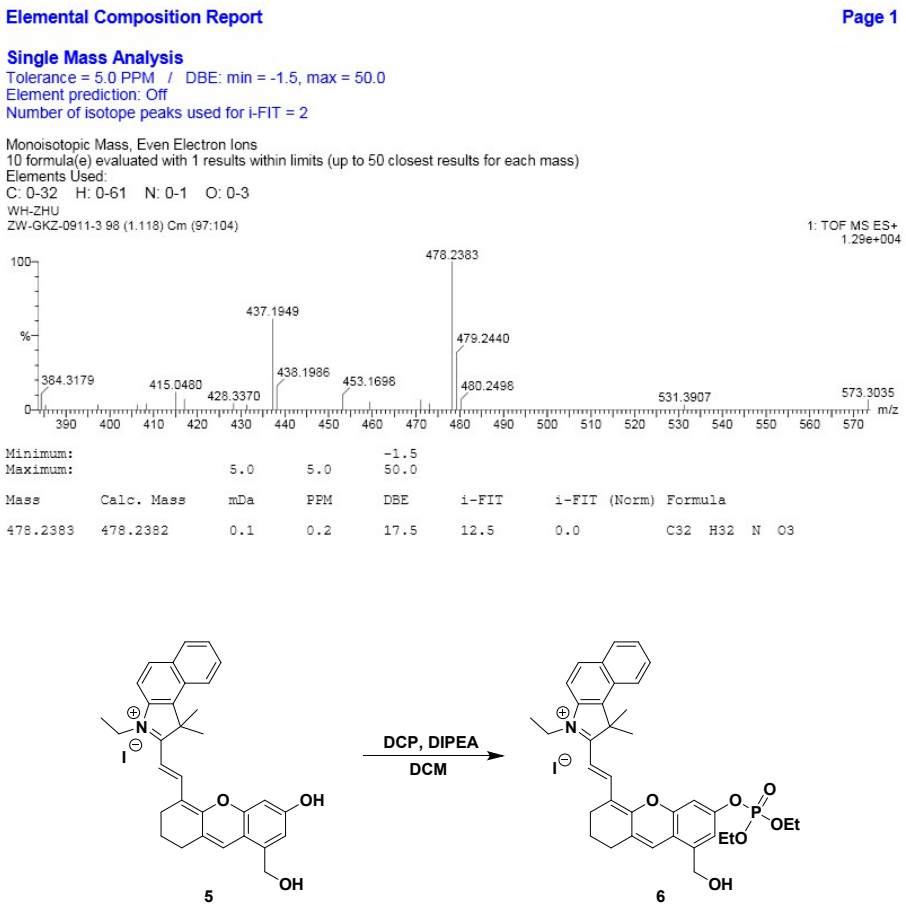
To a 100 mL round bottom flask containing **4** (330 mg, 0.45 mmol) and NEt_3 (1.2 mL, 9.0 mmol) in anhydrous acetonitrile (10 mL), 3,5-dihydroxybenzyl alcohol (126 mg, 0.9 mmol) was added and stirred at 110 °C for 8 h under an Ar atmosphere. The solvent was evaporated, and the residue was purified by column chromatography (CH_2Cl_2:CH_3OH = 100:1 to 50:1) to give **5** as a blue solid (219 mg, 81%). ^1H NMR (400 MHz, CD_3OD): δ = 1.53 (t, *J* = 7.1 Hz, 3H), 1.96 (s, 2H), 2.08 (s, 6H), 2.78 (d, *J* = 22.5 Hz, 4H), 4.48 (q, *J* = 6.8 Hz, 2H), 6.49 (d, *J* = 14.5 Hz, 1H), 6.83 (s, 1H), 6.93 (s, 1H), 7.59 (d, *J* = 7.6 Hz, 1H), 7.63 (s, 1H), 7.83 – 7.71 (m, 3H), 8.07 (d, *J* = 7.8 Hz,

1H), 8.12 (d, J = 8.7 Hz, 1H),8.35 (d, J = 8.4 Hz, 2H), 8.82 (d, J = 14.6 Hz, 1H). HRMS-ESI (m/z): calcd for $C_{32}H_{32}INO_3$, 605.1427, found $[M\text{-}I]^+$, 478.2377.

^1H NMR spectrum of compound **5** in CD_3OD:



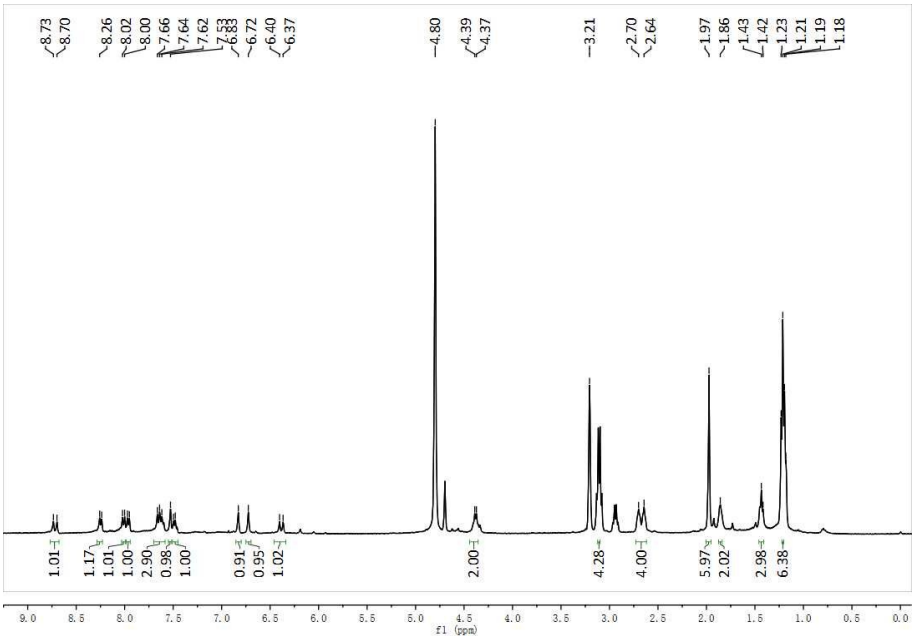
ESI-MS spectrum of compound **5**:



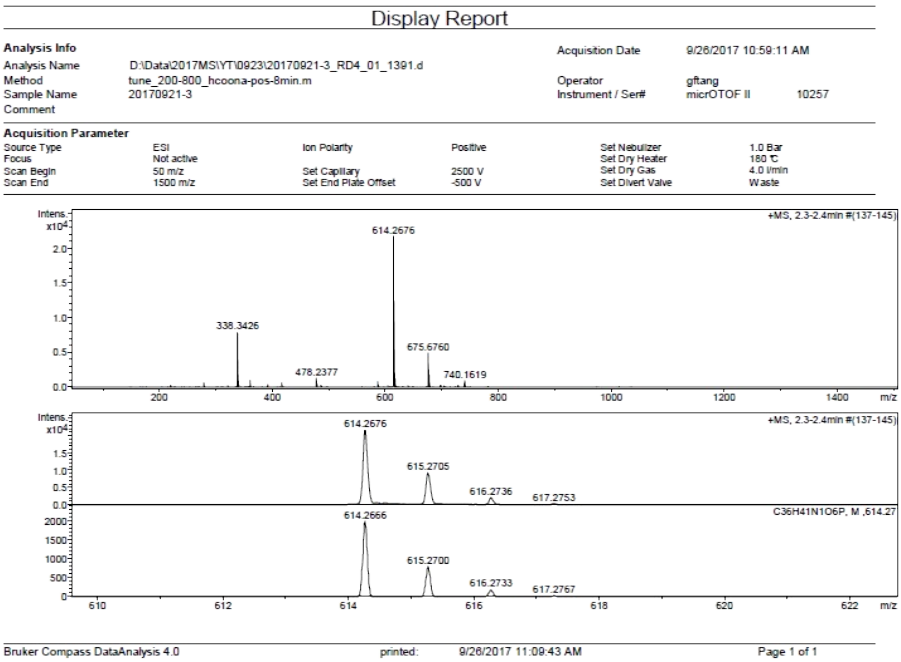
To a solution of compound **5** (140 mg, 0.2 mmol) and DIPEA (380 mg, 2.0 mmol) in anhydrous

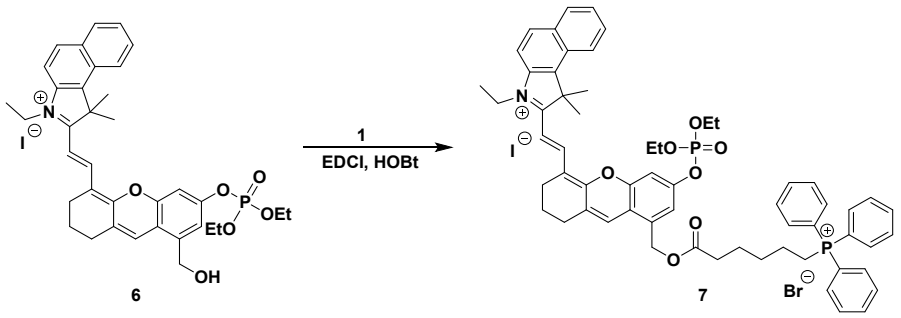
CH_2Cl_2 (10 mL) was added diethyl chlorophosphate, DCP (100 μL, 0.4 mmol). The solution was stirred at room temperature overnight. Then, the solvent was evaporated, and the residue was purified by column chromatography (CH_2Cl_2:CH_3OH = 200:1 to 50:1) to give **6** as a blue solid (133 mg, 89.8%). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.20 (t, *J* = 7.1 Hz, 6H), 1.43 (t, *J* = 7.1 Hz, 3H), 1.86 (s, 2H), 1.97 (s, 6H), 2.67 (d, *J* = 22.5 Hz, 4H), 3.11 (q, *J* = 7.1 Hz, 4H), 4.38 (q, *J* = 6.8 Hz, 2H), 6.38 (d, *J* = 14.5 Hz, 1H), 6.72 (s, 1H), 6.83 (s, 1H), 7.49 (d, *J* = 7.6 Hz, 1H), 7.53 (s, 1H), 7.75 – 7.57 (m, 3H), 7.96 (d, *J* = 7.8 Hz, 1H), 8.01 (d, *J* = 8.7 Hz, 1H), 8.25 (d, *J* = 8.4 Hz, 1H), 8.72 (d, *J* = 14.6 Hz, 1H). HRMS-ESI (m/z): calcd for $C_{36}H_{41}INO_6P$, 741.1716, found $[M-I]^+$, 614.2676.

^{1}H NMR spectrum of compound **6** in CD_3OD:



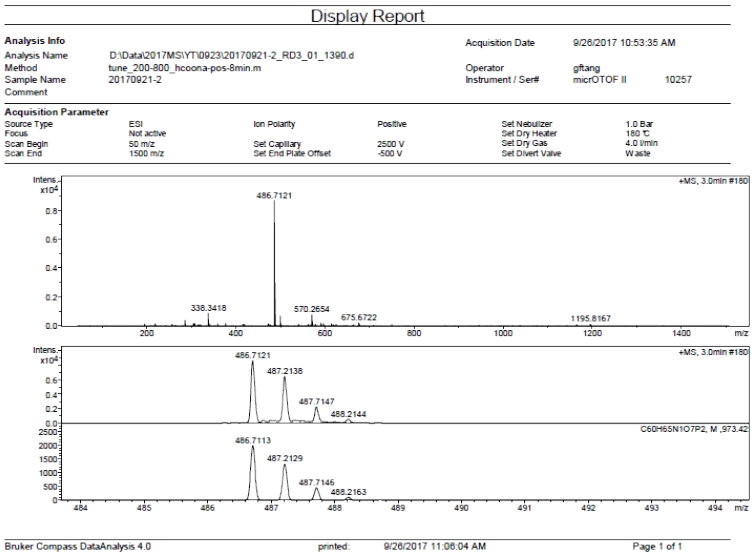
ESI-MS spectrum of compound **6**:

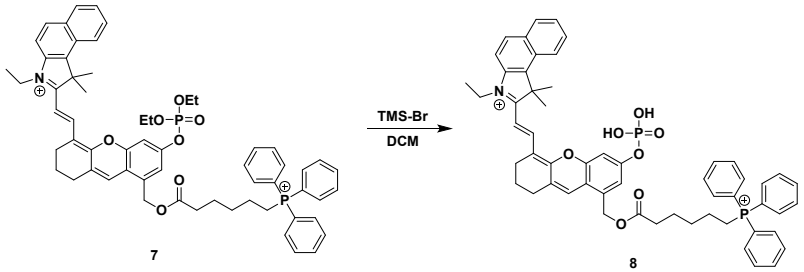




To a solution of compound **6** (150 mg, 0.23 mmol), EDCI (100 mg, 0.46 mmol) and HOBt (10 mg, 0.05 mmol) in anhydrous DMF (10 mL) was added compound **1** (200 mg, 0.46 mmol). The solution was stirred at room temperature for 5 h. Afterwards, the reaction mixture was extracted by CH_2Cl_2 (100 mL) and washed with brine (200 mL), dried with $MgSO_4$, filtered and evaporated to give the crude product, which was further purified by column chromatography on silica gel to obtain **7** (184 mg, 67.9%). HRMS-ESI (m/z): calcd for $C_{60}H_{65}BrINO_7P_2$, 1179.2464, found $[M\text{-}I\text{-}Br]^{2+}$, 486.7121.

ESI-MS spectrum of compound **7**:

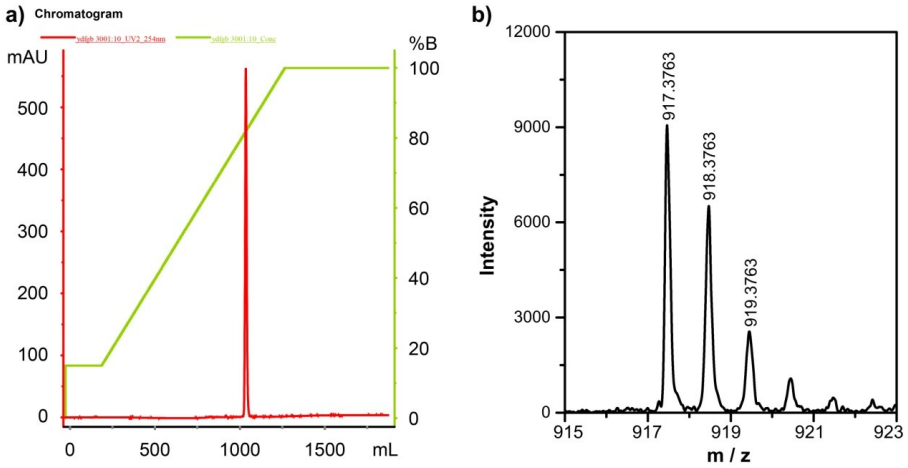




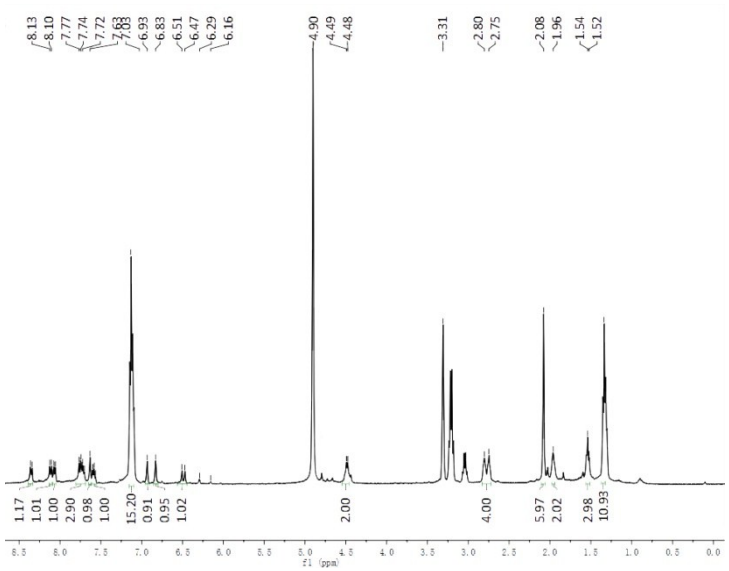
To a solution of compound **7** (56.2 mg, 0.085 mmol) in anhydrous CH_2Cl_2 (5 mL) was added Bromotrimethylsilane, TMS-Br (224 μL, 1.7 mmol). The solution was stirred at room temperature for 24 h. Afterwards, the reaction was quenched by methanol and stirred for 3 h. The organic solvent was evaporated and concentrated under vacuum to obtain oily residue. The crude product was further purified by preparative HPLC. Purity of compound **8** was checked by HPLC on a RP-C18 column using water/MeOH as eluent and characterized by MALDI-TOF mass spectrometer.

Compound **8**: A blue solid (25 mg, 0.03 mmol, Yield: 32.4%, purity HPLC: 99.8%). MALDI-TOF (m/z): calcd for 917.3599, found $[M]^+$ 917.3763.

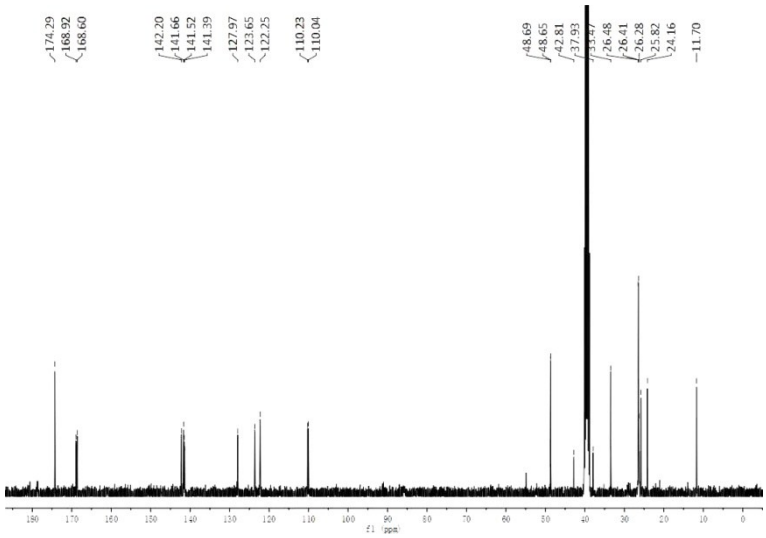
HPLC and MALDI-TOF MS analysis of compound **8**:



^{1}H NMR spectrum of compound **8**:



^{13}C NMR spectrum of compound **8**:



3. UV/Vis and Fluorescence experiments:

Absorption spectra were recorded using a UV-visible spectrometer and fluorescence spectra were measured with fluorescence spectrometer (PerkinElmer LS55) at 25 °C. All spectra were corrected for intensity using the manufacturer-supplied correction factors and corrected for background absorption by subtracting a blank scan of the buffer system. The absorption, and emission spectra of ETP were investigated in PBS (pH 8.0, 25 °C).

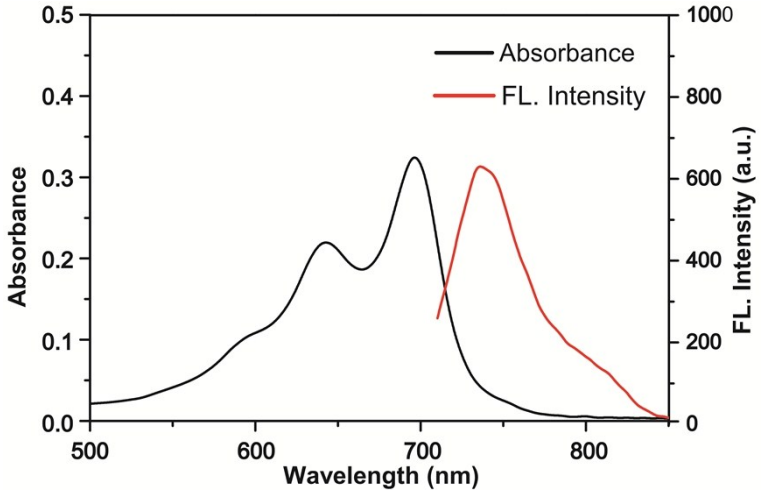


Fig. S1 Absorption and fluorescence spectra of HemiCy in DCM.

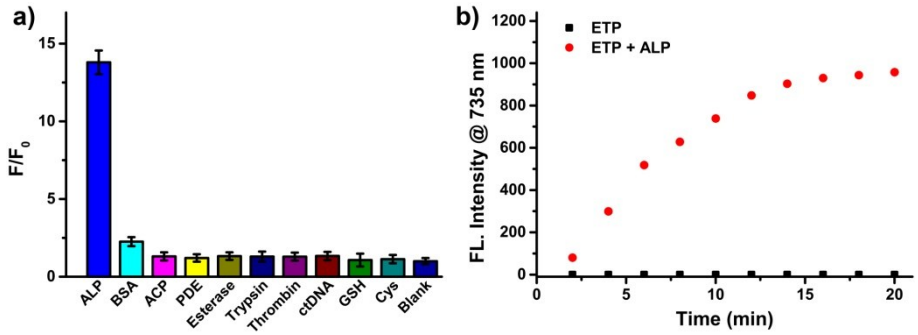


Fig. S2 a) Fluorescence response of ETP (20 μM) at 735 nm (λ_{ex} 710 nm) upon the addition of various species in PBS (pH 8.0, 25°C). b) Time-dependent fluorescence response of ETP (20 μM) at 735 nm (λ_{ex} 710 nm) with or without ALP (0.1 U/mL) in PBS (pH 8.0, 25 °C).

4. Dynamic light scattering (DLS) and Zeta potential:

The DLS and Zeta potential were determined by Nano-ZS (zatasizer, Malvern) instrument, and starting solutions were filtered prior to use. Samples of ETP with or without ALP (0.1 U/mL) in PBS (pH 8.0, 25°C) were tested in a total sample volume of 1.0 mL, respectively.

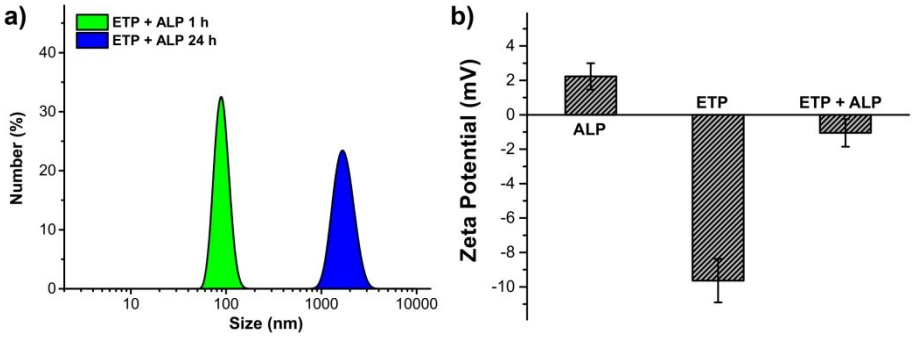


Fig. S3 a) Hydrodynamic size profiles of probe ETP (20 μM) in PBS at 1 h and 24 h after addition of ALP (0.1 U/mL). b) Zeta potentials of probe ETP (20 μM) with or without ALP (0.1 U/mL) in PBS (pH 8.0, 25 °C).

5. Photothermal Property and Photo-stability:

The ETP aqueous solution with or without the addition of ALP was exposed with a laser (650 nm, 0.5 W/cm^2, 5 min), and deionized water was used as a control. During these measurements, the temperature was monitored by a thermal imaging camera. Three cycles of irradiations were also carried out (650 nm for ETP and 808 nm for ICG, 0.5 W/cm^2, 5 min). The initial and max temperatures of samples in each cycle were measured. The UV−vis absorbance were recorded after each irradiation including for ETP (100 μM, 2 mL) and ICG aqueous solution (100 μM, 2 mL).

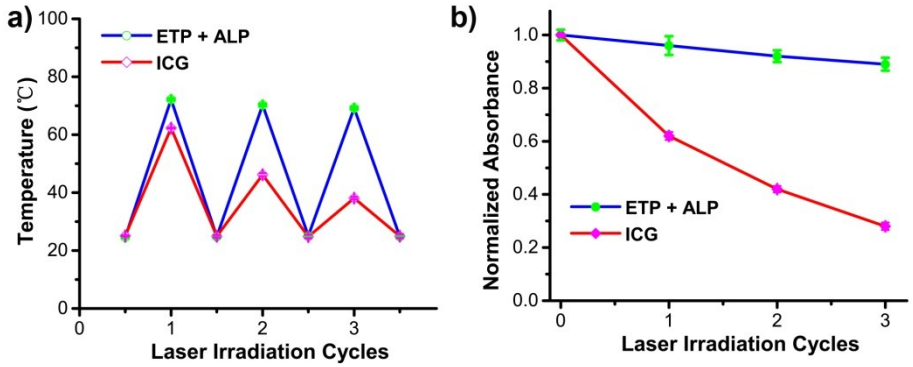


Fig. S4 The thermal curves and b) absorbance intensity change of ETP with ALP and ICG after repeated laser irradiation (n = 3), respectively (the laser wavelength for ETP test is 650 nm and for ICG test is 808 nm).

6. Cell experiments:

Cell culture: The human prostate cancer cell line PC-3 and human breast cancer cell line MCF-7 were purchased from Chinese Academy of Sciences Committee Type Culture Collection Cell Bank (Shanghai, China). PC-3 cells were routinely maintained in RPMI 1640 medium (Gibco) and MCF-7 cells were cultured in DMEM (Gibco) medium. All medium was supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Gibco). Periodic testing ensured the absence of mycoplasma contamination. Cells were passaged using 0.25% Trypsin/EDTA (Gibco) when they reached 80%-90% confluence and seeded for the experiments.

Alkaline phosphatase assay kit: The ALP activity was detected using alkaline phosphatase assay kit according to the manufacturer's instructions.

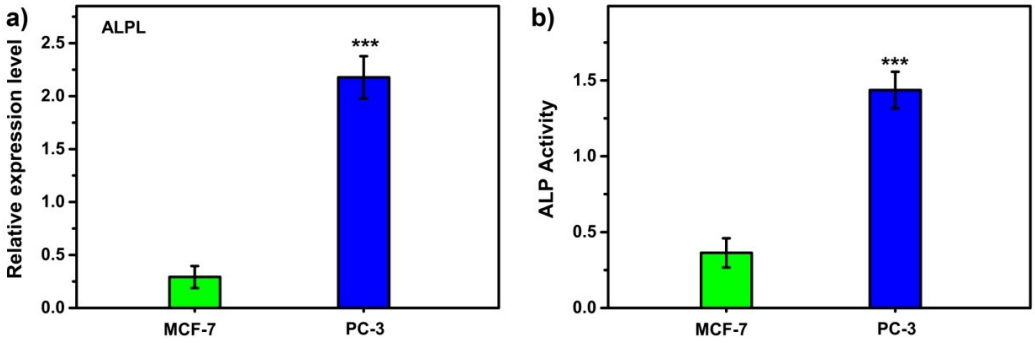


Fig. S5 a) ALPL mRNA expression level and b) activity of ALP in PC-3 and MCF-7 cells. Statistical significance: ***P < 0.001.

Western blot: The proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked by 5% non-fat milk in TBST for 2 h and incubated with primary antibodies at 4 °C overnight. After washing thrice in TBST for 5 min each time, the membrane was incubated with labeled secondary antibody at room temperature for 1 h. The proteins were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and images were acquired with the Bio-Rad System. Antibodies against fibronectin were provided by Abcam. β-actin was used as an internal control.

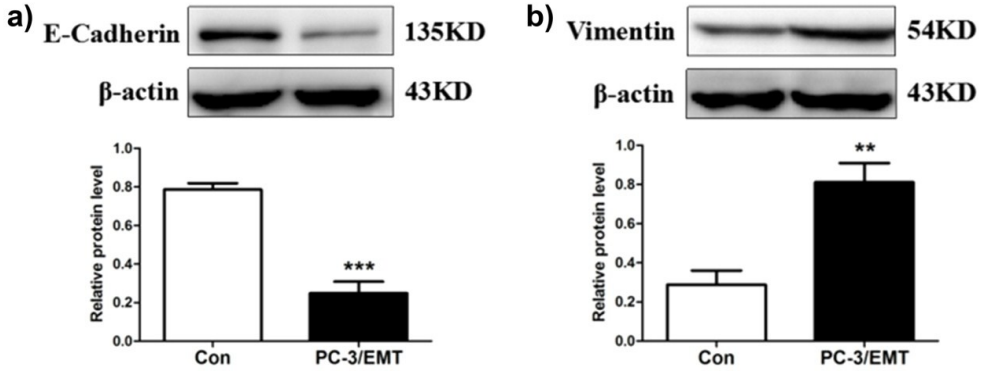


Fig. S6 Representative western blots of a) E-cadherin and b) Vimentin in primary PC-3 cells (control) and metastatic PC-3/EMT cells. Data represent mean ± SD, n = 3. Statistical significance: **P < 0.01.

Confocal microscopic imaging: PC-3 cells were seeded in a 35 mm petri dish with a glass cover slide and allowed to adhere overnight without or with ALP inhibitor (sodium orthovanadate, 50.0 μM) before the administration of ETP. Then the cells were incubated with 20 μM ETP in complete medium for 2 h at 37 °C. After incubation, cells imaging was carried out after washing with PBS twice. Cell fluorescence images were obtained with a confocal laser scanning microscope (Nikon A1, Japan, 60×oil-immersion objective lens). Excitation: 638 nm, emission collected: 664-735 nm.

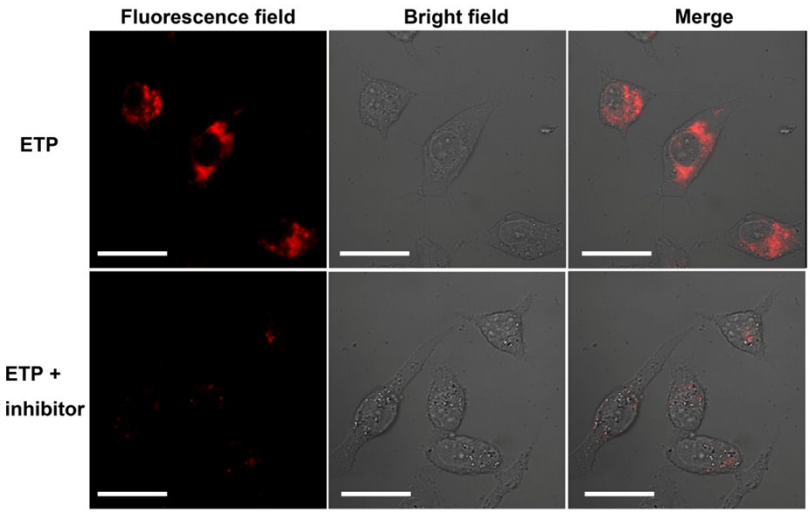


Fig. S7 Confocal laser scanning microscopy images of PC-3 cells treated with or without ALP inhibitor before the administration of ETP, respectively. Excitation: 638 nm, emission collected: 664-735 nm. Scale bar is 20 μm.

7. *In vivo* experiments:

The *in vivo* study was performed according to a protocol approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. A total of 20 male BALB/c nude mice (5 weeks old, 18-20 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China) and maintained in a specific pathogen-free environment. Each mouse was subcutaneously injected with a mixture of 0.2 mL PBS containing 10^7 cells into the right hind flank. Lengths and widths of tumors were measured individually using a vernier caliper. Tumor volumes were calculated using the following formula: tumor volume = length × width^2 × 0.5.

***In vivo* fluorescence imaging:** Mice were imaged using an IVIS spectrum series *in vivo* imaging system (PerkinElmer). The fluorescence intensity from the tumor and other area was integrated. After mice were injected *via* the tail vein with ETP from PBS stock solutions, they were placed in the IVIS spectrum series *in vivo* imaging system (PerkinElmer) and scanned to determine the endogenous signal of tumors (excitation: 710 nm, emission collected: 730-760 nm). Mice were anesthetized by 2% isoflurane inhalation for 30 seconds in anaerobic box.

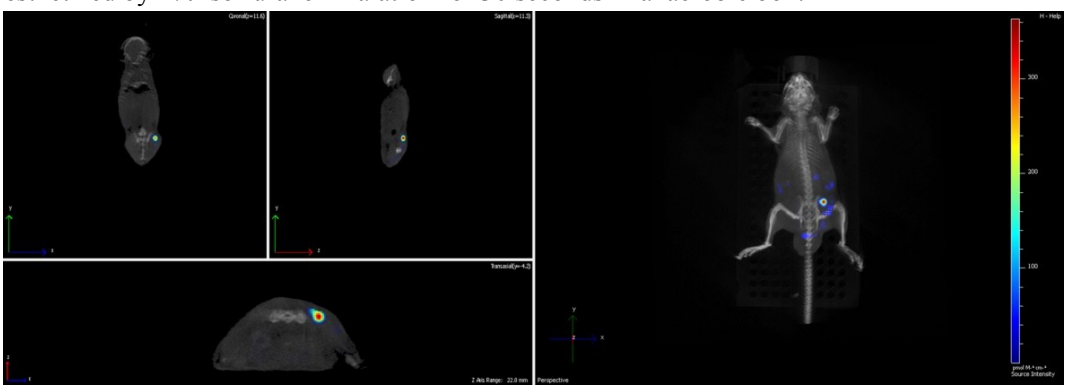


Fig. S8 Three-dimensional NIR image reconstruction of the PC-3 xenograft tumor mouse without inhibitor treatment using an IVIS spectrum series *in vivo* imaging system.

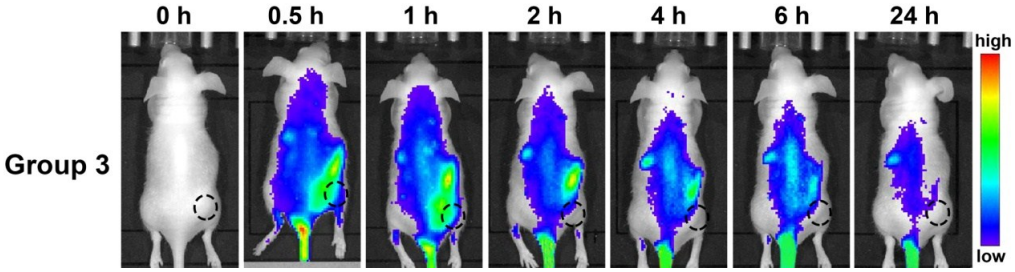


Fig. S9 *In vivo* NIR fluorescence imaging of mice bearing MCF-7 tumors intravenously injected with ETP. Tumors were circled with dashed lines. All images were acquired under the same instrument conditions.

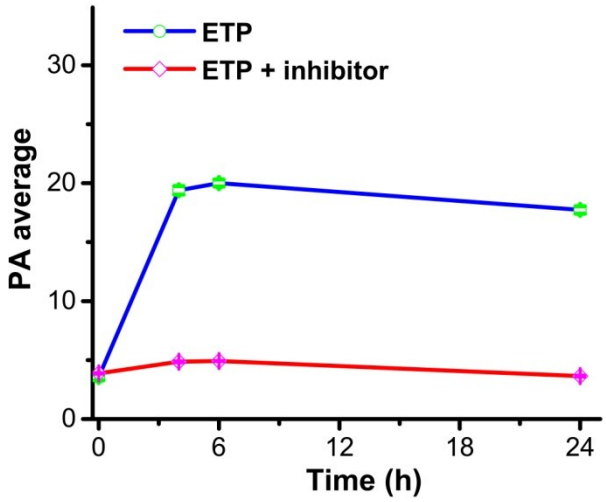


Fig. S10 Averaged PA signal intensity in the tumor area at different times after *i.v.* injections of ETP and ETP with inhibitor.

For *ex vivo* organ imaging: Mice were euthanized by excess isoflurane inhalation and the organs were dissected after *in vivo* imaging experiments. The heart, liver, kidney, lung, stomach, spleen and tumor were obtained from mice injected with ETP; One group of PC-3 xenograft mouse were intravenously injected with ALP inhibitor before the administration of ETP. Fluorescence images of organs were obtained directly using an IVIS spectrum series *in vivo* imaging systems (PerkinElmer).

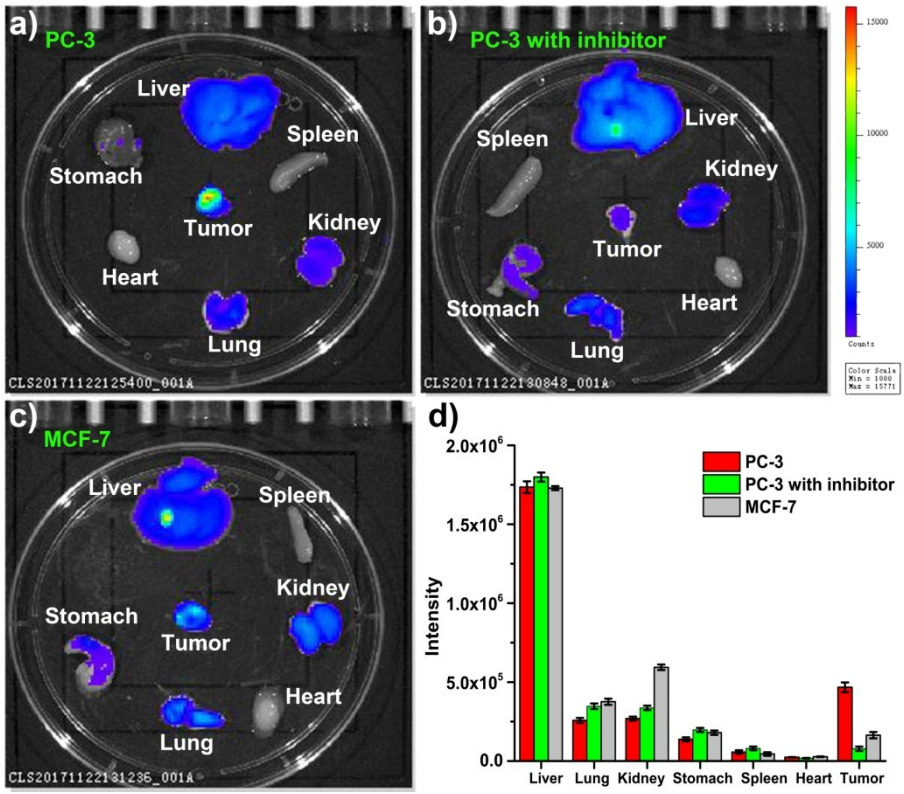


Fig. S11 a-c) *Ex vivo* NIR fluorescence imaging of ETP distribution in isolated organs and tumors of mice bearing a) PC-3 tumor, b) PC-3 tumor with ALP inhibitor or c) MCF-7 tumor after intravenous injection with ETP. d) Quantitative analysis of relative organ accumulation in the xenograft tumor model system mice. Data are represented as average ± standard error (n = 3). PC-3 means mouse bearing PC-3 tumor; PC-3 with inhibitor means mouse bearing PC-3 tumor pre-injected with ALP inhibitor; MCF-7 means mouse bearing MCF-7 tumor.

***In vivo* antitumor studies:** When the tumors reached a mean volume of 100 mm^3 after inoculation of PC-3 cells, The tumor-bearing mice were randomly divided into 4 groups: 1) PBS, 2) PBS with NIR laser, 3) ETP only, and 4) ETP with NIR laser. The power density of 650 nm laser for PTT was 0.5 W/cm^2 and the exposure time was 5 min. During therapy, the tumor volumes and body weights were measured every two days. The mice were sacrificed till 14 d post-treatment according to institutional guidelines. Tumors were resected, weighed, fixed in formalin and embedded in paraffin. The therapeutic efficacy of the treatment was evaluated by the tumor-inhibition rate (TIR). This was calculated using the following equation: TIR (%) = (mean tumor weight of PBS group - mean tumor weight of experimental group)/mean tumor weight of PBS group × 100.

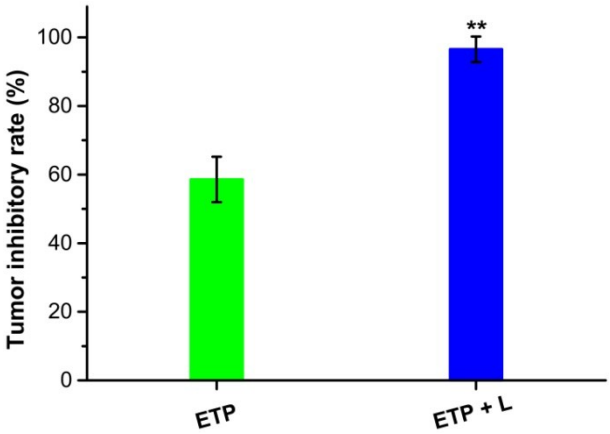


Fig. S12 The tumor inhibitory rate (TIR) after different treatment in PC-3 tumor-bearing mice. Data are represented as average ± standard error (n = 5). L means laser. Statistical significance: **P < 0.01.

For staining of tissue slices: Freshly dissected hearts, livers, spleens, lungs, kidneys and tumors from mice of four groups were fixed and embedded in paraffin. After being cut into 4 μm slices, the sections were deparaffinized and stained with Hematoxylin and Eosin solution. The scan was used with a microscope (Nikon, Japan).